

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF RECEIPT OF
RECORD COPY

(PCT Rule 24.2(a))

From the INTERNATIONAL BUREAU

To:

LEE, Duckrog
2nd Fl., Yeil Bldg.
700-19 Yorksam-dong, Kangnam-gu
Seoul 135-928
RÉPUBLIQUE DE CORÉE

Date of mailing (day/month/year) 19 December 2000 (19.12.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference YL001019BPCT	International application No. PCT/KR00/01183

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY et al (for all
designated States except US)

RHEE, Sangki et al (for US)

International filing date : 19 October 2000 (19.10.00)
Priority date(s) claimed : 19 October 1999 (19.10.99)
Date of receipt of the record copy
by the International Bureau : 08 December 2000 (08.12.00)
List of designated Offices :

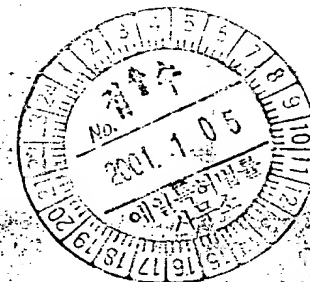
AP : GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW

EA : AM,AZ,BY,KG,KZ,MD,RU,TJ,TM

EP : AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE

OA : BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG

National : AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EE,
ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,
MG,MK,MN,MW,MX,MZ,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,
VN,YU,ZA,ZW



The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer:

Mougamadou ABIDINE
Mougamadou ABIDINE

Facsimile No. (41-22) 740.14.35

Telephone No. (41-22) 338.83.38

PCT REQUEST

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YL001019BPCT

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form - PCT/RO/101 PCT Request Prepared using	PCT-EASY Version 2.91 (updated 10.10.2000)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Korean Industrial Property Office (RO/KR)
0-7	Applicant's or agent's file reference	YL001019BPCT
I	Title of invention	ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM
II	Applicant	applicant only
II-1	This person is:	all designated States except US
II-2	Applicant for	KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY
II-4	Name	52, Oun-dong, Yusong-gu 305-333 Taejon Republic of Korea
II-5	Address:	KR
II-6	State of nationality	KR
II-7	State of residence	82-42-860-4452
II-8	Telephone No.	82-42-860-4594
II-9	Facsimile No.	
III-1	Applicant and/or inventor	applicant only
III-1-1	This person is:	all designated States except US
III-1-2	Applicant for	REALBIOTECH, LTD.
III-1-4	Name	BVC Room #102, KRIBB, 52, Oun-dong, Yusong-gu 305-333 Taejon Republic of Korea
III-1-5	Address:	KR
III-1-6	State of nationality	KR
III-1-7	State of residence	KR

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YL001019BPCT

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III-2	Applicant and/or inventor	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	RHEE, Sangki
III-2-5	Address:	#101, Gadong, Geukdong Villa, Gwangjang-dong, Gwangjin-gu 143-751 Seoul Republic of Korea
III-2-6	State of nationality	KR
III-2-7	State of residence	KR
III-3	Applicant and/or inventor	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	SONG, Kibang
III-3-5	Address:	110-802, Hanbit APT, #99, Oun-dong, Yusong-gu 305-755 Taejon Republic of Korea
III-3-6	State of nationality	KR
III-3-7	State of residence	KR
III-4	Applicant and/or inventor	
III-4-1	This person is:	applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	KIM, Chulho
III-4-5	Address:	2-1101, Shindonga APT, Yongjeon-dong, Dong-gu 300-766 Taejon Republic of Korea
III-4-6	State of nationality	KR
III-4-7	State of residence	KR
III-5	Applicant and/or inventor	
III-5-1	This person is:	applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	RYU, Eunja
III-5-5	Address:	107-1302, Hanbit APT, #99, Oun-dong, Yusong-gu 305-755 Taejon Republic of Korea
III-5-6	State of nationality	KR
III-5-7	State of residence	KR

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YL001019BPCT

III-6	Applicant and/or inventor	
III-6-1	This person is:	applicant and inventor
III-6-2	Applicant for	US only
III-6-4	Name (LAST, First)	LEE, Yongbok
III-6-5	Address:	101-1703, Sangyong APT, Ilgok-dong, Buk-gu 500-160 Gwangju Republic of Korea
III-6-6	State of nationality	KR
III-6-7	State of residence	KR
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent international Authorities as:	agent
IV-1-1	Name (LAST, First)	LEE, Duckrog
IV-1-2	Address:	2nd Fl., Yeil Bldg., 700-19, Yorksam-dong, Kangnam-gu 135-928 Seoul Republic of Korea
IV-1-3	Telephone No.	82-2-555-1717
IV-1-4	Facsimile No.	82-2-555-1784
IV-1-5	e-mail	yeildr@chollian.net
V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CR CU CZ DE DK DM DZ EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW

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V-5	Precautionary Designation Statement In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	Exclusion(s) from precautionary designations NONE		
VI-1	Priority claim of earlier national application		
VI-1-1	Filing date	19 October 1999 (19.10.1999)	
VI-1-2	Number	1999-45302	
VI-1-3	Country	KR	
VII-1	International Searching Authority Chosen Korean Industrial Property Office (KIPO) (ISA/KR)		
VIII	Check list	number of sheets	electronic file(s) attached
VIII-1	Request	5	-
VIII-2	Description (excluding sequence listing part)	46	-
VIII-3	Claims	6	-
VIII-4	Abstract	1	-
VIII-5	Drawings	9	yl001019bpct.txt
VIII-6	Sequence listing part of description	6	-
VIII-7	TOTAL	73	-
VIII-8	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-15	Fee calculation sheet	✓	-
VIII-16	Nucleotide and/or amino acid sequence listing in computer readable form	-	separate diskette
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	Figure of the drawings which should accompany the abstract	5	-
VIII-19	Language of filing of the international application	English	
IX-1	Signature of applicant or agent		
IX-1-1	Name (LAST, First)	LEE, Duckroo	



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10-1	Date of actual receipt of the purported international application	
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YL001019BPCT

10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/KR
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: LEE, Duck Rog 2nd Fl., Yeil Bldg., 700-19, Yorksam-dong, Kangnam-gu, Seoul 135-928, Republic of Korea
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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference YL001019BPCT	Date of mailing (day/month/year) 12 MARCH 2001 (12.03.2001)
International application No. PCT/KR00/01183	International filing date (day/month/year) 19 OCTOBER 2000 (19.10.2000)
Applicant KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY et al	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

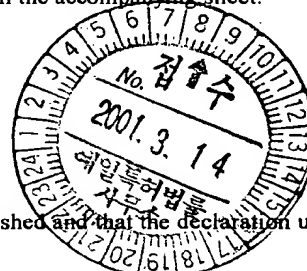
Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland
 Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.



2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:**
☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

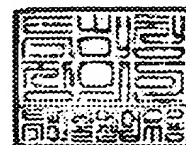
4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/KR Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer COMMISSIONER Telephone No. 82-42-481-5131
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference YL001019BPCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/KR00/01183	International filing date (<i>day/month/year</i>) 19 OCTOBER 2000 (19.10.2000)	(Earliest) Priority Date (<i>day/month/year</i>) 19 OCTOBER 1999 (19.10.1999)
Applicant KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY et al		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☒ Unity of invention is lacking (See Box II).

4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawing to be published with the abstract is Figure No. 5

☒ as suggested by the applicant.

☐ None of the figures.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR00/01183

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

Group I, claims 1-9, drawn to a novel bacteria (K2032) containing levan fructotransferase, novel genes and amino acids coding for levan fructotransferase, expression vectors carrying the genes, and transformed cells with the expression vectors

Group II, claims 10-14, 18-24, drawn to a process of producing difructose dianhydride IV

Group III, claims 15-17, drawn to a process of preparing levan fructotransferase

Since the abovementioned groups of claims do not share any of the technical features identified, a technical relationship between the inventions does not exist. Accordingly, the claims do not relate to one invention or to a single inventive concept, a priori.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any addition fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/KR00/01183
A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C12N 1/20**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 1/20, 15/09, 9/10 ; C12P 19/12, 19/14

 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and Applications for inventions since 1975

 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
IBM, PAJ
C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TANAKA K, KARIGANE T, YAMAGUCHI F, NISHIKAWA S and YOSHIDA N "Action of Levan Fructotransferase of Arthrobacter Ureafaciens on Levanoligosaccharides" In : J. Biochem., vol. 94 (5), Nov 1983, p.1569-78 See the whole document	10
Y	TANAKA K, KAWAGUCHI H, OHNO K and SHOHJI K "Enzyme Formation of Diffructose Anhydride IV from Bacterial Levan" In : J. Biochem., vol. 90 (5), Nov 1981, p.1545-8 See the whole document	10
A	US 5,057,418 (Mitsubishi Kasei Corporation) 15 Oct 1991 See the abstract	10
X	JP 01091777 A (Maruzen Kasei Co. Ltd.) 11 Apr 1989 See the whole document	15
X	JP 01917193 A (Maruzen Kasei Co. Ltd.) 11 Apr 1989 See the whole document	15
Y	JP 11069978 A (Nippon Oil Co. Ltd.) 16 March 1999 See the whole document	15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 MARCH 2001 (02.03.2001)

Date of mailing of the international search report

09 MARCH 2001 (09.03.2001)

 Name and mailing address of the ISA/KR
Korean Industrial Property Office

Facsimile No.

Authorized officer

AHN. Mi-Chung

Telephone No.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR00/01183

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TANAKA K, OI S and YAMAMOTO T "Synthesis of Levan by Levansucrase. Some factors affecting the rate of rate of synthesis and degree of polymerization of levan" In : J. Biochem., vol. 85 (1), Jan 1979, p.287-93 See the whole document	18
Y	US 4,879,228 (IGI Biotechnology, Inc.) 15 Oct 1991 See the abstract	18, 19
Y	KOYNO K, YANASE H, TONOMURA K, KAWASAKI H and SAKAI T "Cloning and characterization Zymomonas mobilis genes encoding extracellular levansucrase and invertase" In : Biosci Biotechnol Biochem, vol. 59 (2), Feb 1995, p.289-93 See the abstract	19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR00/01183

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,057,418	15.10.91	JP 1225492A2 EP 332108B1 DK 107589A DE 68915584T2 CA 1329161A1	08.09.89 01.06.94 08.09.89 12.01.95 03.05.94
US 4,879,228	07.01.89	WO 8604091A1 US 4863719 US 4769254 JP 62501745T2 IL 77526A1 EP 208717A1	17.07.86 05.09.89 06.09.88 16.07.87 10.09.89 21.01.87
JP 01091793	11.04.89	JP 2614460B2	28.05.97
JP 01091777	11.04.89	JP 2561100B2	04.12.96
JP 11069978	16.03.99	NONE	

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29185 A1

(51) International Patent Classification⁷: C12N 1/20

Sangyong Apt., Ilgok-dong, Buk-gu, Gwangju 500-160 (KR).

(21) International Application Number: PCT/KR00/01183

(22) International Filing Date: 19 October 2000 (19.10.2000)

(74) Agent: LEE, Duckrog; 2nd Fl., Yeil Bldg., 700-19 Yorksam-dong, Kangnam-gu, Seoul 135-928 (KR).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1999/45302 19 October 1999 (19.10.1999) KR

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(71) Applicants (*for all designated States except US*): KO-REA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY [KR/KR]; 52, Oun-dong, Yusong-gu, Taejon 305-333 (KR). REALBIOTECH, LTD. [KR/KR]; BVC Room #102, KRIBB, 52, Oun-dong, Yusong-gu, Taejon 305-333 (KR).

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

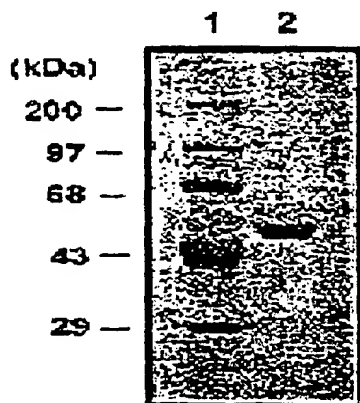
(75) Inventors/Applicants (*for US only*): RHEE, Sangki [KR/KR]; #101, Gadong, Geukdong Villa, Gwangjang-dong, Gwangjin-gu, Seoul 143-751 (KR). SONG, Kibang [KR/KR]; 110-802 Hanbit Apt., #99, Oun-dong, Yusong-gu, Taejon 305-755 (KR). KIM, Chulho [KR/KR]; 2-1101, Shindonga Apt., Yongjeon-dong, Dong-gu, Taejon 300-766 (KR). RYU, Eunja [KR/KR]; 107-1302, Hanbit Apt., #99, Oun-dong, Yusong-gu, Taejon 305-755 (KR). LEE, Yongbok [KR/KR]; 101-1703,

Published:

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM



(57) Abstract: Disclosed is the production of difructose dianhydride IV from sugar. A sugar solution is subjected to reaction at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase derived from *Z. mobilis* to produce levan. The levansucrase is prepared by culturing *E. coli* BL21 (DE3)/pEL12 (KCTC 8661), harvesting and homogenizing the cells, and isolating levansucrase from the cell homogenate. Levan is purified from the reaction solution and subjected to reaction at 25-50 °C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV. The levan fructotransferase is obtained from *E. coli* JUD81 (KCTC 0877BP). Also, disclosed are a gene coding for the levan fructotransferase and an expression vector pUDAF81 carrying the gene.

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ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV
FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR
THEM

TECHNICAL FIELD

The present invention relates to the enzymatic production of difructose dianhydride IV (hereinafter referred to as "DFA IV") from sucrose. More particularly, the present invention relates to enzymes which take part in the
5 production of DFA IV from sugar and their use. In addition, the present invention relates to the production of the intermediate product during the production of DFA IV. Also, the present invention is concerned with novel genes coding for the enzymes, expression vectors carrying the genes, and transformed cells with the expression vectors.

10 BACKGROUND ART

Difructose dianhydride was first found in 1929 by Jackson et al. by analyzing a by-product which was produced when inulin was treated with sulfuric acid to prepare a fructose syrup. Difructose dianhydride, a kind of a cyclic disaccharide, consists of two fructose residues in which a reducing end of
15 each residue is linked to a non-reducing hydroxy group of the counter residue. There have been discovered five kinds of difructose dianhydride, named DFA I to DFA V, thus far. Of them, DFA II and V are found to be synthesized only chemically while the others can be produced enzymatically: DFA I and III are produced from inulin by the action of inulin fructotransferase; and DFA IV from
20 levan by the action of levan fructotransferase.

Difructose dianhydride is a non-digestive, non-fermentative sub-saccharide which is not digested in the animal body. In addition to being useful as a low-calorie sweetener, the sub-saccharide plays a role in inhibiting tooth decay and as a productive factor for *Bifidus* bacteria. It is also reported that
5 difructose dianhydride is used as an absorption factor of minerals in the body (Baik, B. H, Lee, Y. W., and Lee, Y. B.; U.S. Pat. No. 5,700,832, UK. Pat. No. GB 2 308 547 A, Japanese Pat. Appl'n No. 8-51370, Korean Pat. Laid-Open Publication No. 96-13376, Sakurai et al., 1997).

Representative of polyfructans, inulin and levan, both naturally occurring
10 fructose homopolysaccharides, are used to prepare difructose dianhydride (Han, 1989). From them, difructose dianhydride can be synthesized chemically or enzymatically. Chemical synthesis of difructose dianhydride from the natural polyfructans, however, suffers from significant disadvantages. For example, chemical synthesis is of low reaction selectivity, followed by complicated
15 separation and purification. What is worse, it produces pollution of the environment. Consequently, these problems do not vest economical production value in the chemical synthesis. In contrast, enzymatic synthesis using biocatalysts, such as microbes or enzymes, is now regarded as being very economically favorable in synthesizing difructose dianhydride from the natural
20 polyfructans.

Since the discovery of difructose dianhydride III synthase (inulin fructotransferase) from microbes by Tanaka in 1972, enzymes that have the function of synthesizing difructose dianhydrides have been isolated from several microbial sources. The difructose dianhydrides which can be synthesized by
25 such microbe-derived enzymes include DFA I, III and IV. DFA IV is synthesized from levan by the catalytic action of levan fructotransferase and two microorganisms, *Arthrobacter ureafaciens* and *Arthrobacter nicotinovorans* GS-

9 are found to produce DFA IV.

Only a very small amount of levan fructotransferase is synthesized from these bacteria and, thus, its use in the production of difructose dianhydride is very unfavorable in terms of technical and economical aspects. Now generally,
5 in order to obtain a large amount of a gene of interest, a gene recombinant technique is employed. That is, a levan fructotransferase gene is first isolated from its microbial source and cloned, followed by mass-expression in *E. coli*. Of the levan fructotransferase-producing strains discovered so far, only *A. nicotinovorans* GS-9 is achieved in cloning its levan fructotransferase gene.

10 Being a substrate of levan fructotransferase to produce DFA IV, levan, a homopolysaccharide of fructose, is prepared from sucrose by the transfructosylation reaction of levansucrase. The enzymes which can catalyze the hydrolysis of sucrose are exemplified by sucrase (beta-D-fructofuranosidase, EC 3.2.1.26), levanase (beta-2,6-D-fructan fructanohydrolase, EC 3.2.1.65),
15 levansucrase (beta-2,6-fructan: D-glucose-1-fructosyl transferase, EC 2.4.1.10), maltase (alpha-D-glucoside glucohydrolase, EC 3.2.1.20), etc. Levan can be produced from sucrose by taking advantage of the transfructosylation activity of levansucrase (Tanaka & Yamamoto, *J. Biochem.*, 85, 287 (1979)).

There are disclosed methods for the production of levan using
20 levansucrase. For example, it is described in U.S. Pat. No. 4,879,228 and International Publication No. WO 86-4091 that levan is produced by a fermentation process in which advantage is taken of the microbes employing sucrose in their metabolism. The patents, however, suffer from many problems. There is required a lengthy culture period to produce levan.
25 Further, because the culture contains various products, a difficult purification procedure is needed, giving rise to a decrease in the production yield of levan. When account is taken of these problems, an enzyme reaction process has an

advantage over the culture process in that, because the products of levansucrase are dependent on the reaction conditions for the production of levan from sucrose, desirable products can be obtained by controlling the conditions with ease.

5 It is reported that levan finds numerous applications in the medicinal field, such as a serum substituent (Dedonder *et al.*, *Bull. Soc. Chim. Biol.*, 39, 438 (1957); Schechter *et al.*, *J. Lab. Clin. Med.*, 61, 962(1963)), a colloid stabilizer, an immune agent, a pharmacological enhancer, etc. (Leibovici *et al.*, *Anticancer Res.*, 5, 553 (1985); Stark *et al.*, *Br. J. Exp. Path.*, 67, 141 (1986)),
10 the foodstuffs field, such as quality improver, a stabilizer, an additive for health food, etc. (Hatcher *et al.*, *Bioprocess Technol.*, 11, 1(1989)), the typographic field, and the cosmetic field (Whiting *et al.*, *J. Inst. Brew.*, 73, 422 (1967); Han, *Bioprocess Technol.*, 12, 1(1920)).

15 DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide an enzymatic process for producing levan from sugar at a high yield.

It is another object of the present invention to provide a levansucrase gene derived from *Z. mobilis*, which contains such a specific tool as to allow its
20 protein to be purified with ease.

It is a further object of the present invention to provide a recombinant expression vector carrying a levansucrase gene derived from *Z. mobilis*, which can express levansucrase in *E. coli*.

It is still a further object of the present invention to provide a bacterial
25 species which is transformed by the recombinant expression vector and can produce levansucrase.

It is yet a further object of the present invention to provide a process of preparing levansucrase by taking advantage of the transformed bacteria.

It is still another object of the present invention to provide an enzymatic process for producing DFA IV from levan at a high yield.

5 It is yet another object of the present invention to provide a novel bacteria *A. ureafaciens* K2032, which produces levan fructotransferase.

It is yet another object of the present invention to provide a novel gene coding for levan fructotransferase, derived from *A. ureafaciens* K2032.

10 It is yet another object of the present invention to provide a recombinant levan fructotransferase gene, which contains such a specific tool as to allow its protein to be purified with ease.

It is yet another object of the present invention to provide a recombinant expression vector carrying the recombinant gene and a bacterial species which anchors the recombinant expression vector.

15 It is yet another object of the present invention to provide a process of preparing levan fructotransferase by taking advantage of the transformed bacteria.

20 It is yet another object of the present invention to provide an enzymatic process for producing DFA IV from sucrose at a high yield by utilizing the levansucrase and the levan fructotransferase.

In accordance with a first embodiment of the present invention, there is provided a process for producing levan from sugar, in which a sugar solution is subjected to reaction at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase derived from *Z. mobilis*. In one version of
25 this embodiment, the reaction is carried out at 0-15 °C and the sugar solution has a sugar concentration of 10-30 % (w/v).

In accordance with a second embodiment of the present invention,

there is provided a novel levansucrase gene of *Z. mobilis*, which has the following base sequence:

1 GCGGTGACCCCGACTTCCCTCGACGACCACCGTCCCTACCGGCCGACCGCCGGGCCGACTGCTCCTCAGCCTAGACGGGCCCTC
5 91 CTCGAGGTCTTCGTGCGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGGCCGGCGGTGTGACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGCGATCGACGTGAGGCGCCAGCGATGTGACGCCCTGAACCTGCCCGCTTCTGGGCTGACGA
271 GCGCTCCACCCCGACAGCTCTCTTCTACCGTGTCCCGAACAGGGTGGACGCTTCGTGCGCCCAACCGTCCACGAGAGGAACAGCA
361 ATGACGCGCGCATCTCAGCGCGCGTGTCTCAGGGAGCGCGCGCGAGCACTCGCCCTGATCTTGGCGGTGCTGTCCGCGCTGCA
451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCAACGCCCGGTCAAC
10 541 ACCCAGGCGCTACCGAGTGTACTACCTGCACTCCGACCAGAACAACGGCCCCGGCGGTGGGACCACGCGAGCAGACCGCAGCGGCTC
631 GCCTTCACGCACACCGCACCGTGATGCCGTGCGGCCGACTTCCCGTGTGGTCCGGGTCGGCGGTCTGCGCACCGCGAACACGGCA
721 GGGTTGCGCGCGCGCGGTGCTGCGCTCGCGACCCAGCGACCGGCGTCCGCAAGTACCAGGAGCAGTACCTCTACTGGTCGACC
811 GACGGCGGGTTCAGTTACCGCCCTGCCGACCCCGTCACTGTAACAACGACGGTCGCGCGCCACCAACGCCCGCGAGATCGAGAAC
901 GCCGAGTGGTCCGCGACCCCAAGTCACTGGGACACCGCCCGGGAGAATGGGTCTGCGTCACTGGACGACTCGGTCACCCCGCTC
15 991 TACACCTCGCCGAACCTGCGCGACTGGACACTTCGCCGCAACTTCGACTACCCGAACACGCCCCTCGGCGGCATCGAGTGCCCGACCTG
1081 TTCGAGATCACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCTACGGCATCGGCCTCCCATGACGTACGCC
1171 TACTGGACAGGCACTGGGACGGCGAGCAGTTCCACGCCGACGACCTACCCCGCAATGGCTCGACTGGGGCTGGGACTGGTACGCGGCC
1261 GTCACCTGGCCATCGATCGACGCGCCCGAGACCAAGCGCCTCGCCATCGCGTGGATGAACAACGGAAGTACGCGCAGCGGACGTCCCC
1351 ACCGACGCATCCGACGGCTACAACGGCGAGAACTCGATCGTCCGCGAGCTCGGGCTCGCCCGACAGCCTGGCGGCTGGTACACCTCTCTG
20 1441 AGCACCCCGTGGCAGCGCTGACGAACTACGTACCGGCCACCAACCACTCCCCGACCGGACCGTTCGACGGCAGCGCCGCTCTGCCATGG
1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCCTGGGACACCGCGACGAACGTTCGCGATCTCGGTGGGCGCTCCCCGACGGAACC
1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGAGGACCTCCGACCTCGCCGGGTACTCGCTCGCCCCCTACTCG
1711 CGAGCGCGCGCCCCATCGACCCCGGCGCGGATCCGTGCACTGCGCATCTCTGTGACACCCAGAGCGTTCGAGGTCTTCGTCAACGCC
1801 GGCCACACCGTGCTCTCCAGCAGGTCCACTTCGCCGAGGCGACACGGGAATCTCGCTTACACCGACGGCGGCCCGCACACTTCACC
25 1891 GGCATCGTCGTCGCGAGATTGGCCAGCGGATCTAGCGATGCACACCAACCGCTCACCGAAGCCGCGCCCCGGGAGACGACGGCCGAC
1981 AATCGACACGTCTCTGTCGTT

In accordance with a third embodiment of the present invention, there is provided a levansucrase of *Z. mobilis*, which has the following amino acid
30 sequence:

1 M L N K A G I A E P S L W T R A D A M K V H T D D P T A T M
31 P T I D Y D F P V M T D K Y W V W D T W P L R D I N G Q V V

61 S F Q G W S V I F A L V A D R T K Y G W H N R N D G A R I G
 91 Y F Y S R G G S N W I F G G H L L K D G A N P R S W E W S G
 121 C T I M A P G T A N S V E V F F T S V N D T P S E S V P A Q
 151 C K G Y I Y A D D K S V W F D G F D K V T D L F Q A D G L Y
 5 181 Y A D Y A E N N F W D F R D P H V F I T P K I G K T Y A L F
 211 E G N V A M E R G T V A V G E E E I G P V P P K T E T P D G
 241 A R Y C A A A I G I A Q A L N E A R T E W K L L P P L V T A
 271 F G V N D Q T E R P H V V F Q N G L T Y L F T I S H H S T Y
 301 A D G L S G P D G V Y G F V S E N G I F G P Y E P L N G S G
 10 331 L V L G N P S S Q P Y Q A Y S H Y V M T N G L V T S F I D T
 361 I P S S D P N V Y R Y G G T L A P T I K L E L V G H R S F V
 391 T E V K G Y G Y I P P Q I E W L A E D E S S N S A A A L S L
 421 L N K *

15 In accordance with a fourth embodiment of the present invention, there is provided a recombinant expression vector, carrying the levansucrase gene.

In accordance with a fifth embodiment of the present invention, there is provided a novel bacteria *E. coli* BL21 (DE3)/pEL12 (KCTC 8661), in which the plasmid carries a levansucrase gene derived from *Z. mobilis*.

20 In accordance with a sixth embodiment of the present invention, there is provided a process of preparing levansucrase, comprising the steps of culturing a bacterial species anchoring a levansucrase gene-carrying, expression plasmid, harvesting and homogenizing the cells, and isolating levansucrase from the cell homogenate. In one version of this embodiment, the levansucrase gene has a
 25 base sequence stretch encoding histidine residues at its 5'- or 3'-end. In another version of this embodiment, the isolating step is carried out using metal ion-affinity chromatography and the bacterial species is *E. coli* BL21(DE3)/pEL12 (KCTC 8661P).

In accordance with a seventh embodiment of the present invention,
 30 there is provided a novel microorganism *A. ureafaciens* K2032, which shows an

activity of selectively producing difructose dianhydride IV from levan and an activity of degrading levan.

In accordance with an eighth embodiment of the present invention, there is provided a novel levan fructotransferase of the following amino acid

5 sequence 1:

1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V
10 91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A
121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T
151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N
181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F
211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L
15 241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A
271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A
301 V T W P S I D A P E T K R L A I A W M N N W K Y A A R D V P
331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L
361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W
20 391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T
421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S
451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
511 G I V V R E I G Q A I *

25

In accordance with a ninth embodiment of the present invention, there is provided a novel levan fructotransferase gene of sequence 2:

1 CGGTGCACCCGACTTCCCTCGACGACCACCGTCCCCCTACCGGCCGACCGCCCGGCGGCGACTGCTCCTCAGCCTAGACGGGCCCTC
30 91 CTCGAGGTCTTCGTCGGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCCTGCGGGCCGGCGGTGTGACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGGATCGACGTGAGGCGGCCAGCGATGCTGACGCCCTGAACCTGCCGCGTCTTGGGCTGACGA
271 GCGCTCCACCCCGACAGCTCTCCTTCTACCGCTGCCGAACAGGGTGGACGCTTCGTCGCGCCACCCGTCCACGAGAGGAACAGCA
361 ATGACGCCGCCATCTCACGCCGCGCGGTGCTCCAGGAGCCGGCGCCGAGCACTCGCCCTGATCTTCGGCGGTGCTGTGCCGCTGCA

451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTCAACC
 541 ACCCAGCGCGCTACCACTGTACTACCTGCACTCCGACCAGAACAAACGGCCCCGGCGGTGGGACCAACGCGAGCACGACCGACGGCGTC
 631 GCCTTCACGCACACCGGACCGTGATGCCGCTCGGGCCGACTTCCCCGTGTGGTCCGGTCCGGCGGTCTCGGCACCGCGAACACGGCA
 721 GGGTTCCGGCGCGGGCGGGTCTGTCGCGCTCGCGACCCAGCCGACCGACGGCGTCCGCAAGTACCAGGACAGTACCTCTACTGGTCGACC
 5 811 GACGGCGGGTTACGTTACCGCCCTGCCGACCCCGTCATCGTCAACACCGACGGTCCGCGCCGCCACACGCCCCCGAGATCGAGAAC
 901 GCCGAGTGGTTCGCGGACCCCAAGATCCACTGGGACACCGCCCCGGGAGAATGGGTCTGCGTCACTCGGACGACTGCGGTACGCGCGTTC
 991 TACACCTCGCGCAACTGCGCGACTGGACACTTCGCGCAACTTCGACTACCCGAACACGCCCCCTCGCGGCGATCGAGTGCCCCGACCTG
 1081 TTCGAGTACACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCTACGGCATCGGCCTCCCATGACGTACGCC
 1171 TACTGGACAGGCACCTGGGACGGCGAGCAGTTCCACGCCGACGACCTACCCCCGAATGGCTCGACTGGGGCTGGGACTGGTACGCGGCC
 10 1261 GTCACCTGGCCATCGATCGACGCGCCGAGACCAAGCGCCTCGCCATCGCTGGATGAACAACTGGAAGTACGCCGACGCGACGTCCCC
 1351 ACCGACGCATCCGACGGCTACAACGGGCAGAACTCGATCGTCCGCGAGCTCGGGCTCGCCCGACAGCCTGGCGGTGGTACACCTCCTG
 1441 AGCACCCCGTGGCAGCGCTGACGAACTACGTACCGCCACCAACCACTCCCCGACCGGACCGTCCGACGGCAGCGCGTCTCGCATGG
 1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCCTGGGACACCGCGACGAACGTGGCATCTCGGTGGGCGCTCCCCGACGGAACC
 1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGGAGGACCTCCGACCTCGCGGGTACTCGCTCGCCCCCTACTCG
 15 1711 CGAGCGCGCGCCCCATCGACCCCGCGCCGATCCGTGCACCTGCGCATCTCGTCCGACACCCAGAGCGTCCGAGGTCTTCGTCACCGCC
 1801 GGCCACACCGTGCTCTCCAGCAGGTCCACTTCGCGGAGGGCGACACGGGAATCTCGCTCTACCCGACGGCGGCCCGCACACTTCAAC
 1891 GGCATCGTCTCGCGGAGATTGGCCAGGCGATCTAGGCGATGCACACCACACCGCTCACCGAAGCCGCGCCCGGGAGACGACGGCGGAC
 1981 AATCGACACGTCTCTCGTCTT

20 In accordance with a tenth embodiment of the present invention, there is
 provided a novel levan fructotransferase gene of sequence 3:

1 GCGGTGCACCCGACTTCCCTCGACGACCACCGTCCCCCTACCGGCCGACCGCCCGGCCGACTGCTCTCAGCCTAGACGGGCCCCCTC
 Aval Aval
 25 91 CTCGAGGTCTTCTCGGGGACGGTGAGGCGACTGCGTGAACCTGGTCTCTGGGGGCGCGGTGTGACCGCGAGCCTCGAGACGGCA
 181 CGGCCAGGAACCGTGACGTGACCGCGATCGACGTGAGGGCGCCAGCGATGCTGACGCCCTGAACCTGCGCGCGTTCTGGGTGACGA
 271 GCGTCCCACCCGACAGCTCTCTTACCGTCCCGGAACAGGGTGGACGCTTCGTGCGGCCACCGCTCCAGAGAGGAACACGCA
 PstI
 361 ATGACGCCGGCATCTACGCCGCGCGTGTCTCAGGAGCGCGCGCGGAGCACTCGCCCTGATCTTCGGCGGTGCTGTGCCGCTGCA
 30 1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
 451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTCAAC
 31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
 541 ACCCAGCGCGCTACCACTGTACTACCTGCACTCCGACCAGAACAAACGGCCCCGGCGGTGGGACCAACGCGAGCACGACCGACGGCGTC
 61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V

In accordance with an eleventh embodiment of the present invention, there is provided a recombinant expression vector, carrying the levan fructotransferase gene.

In accordance with a twelfth embodiment of the present invention, there is provided a process for producing DFA IV from levan, in which a levan solution is subjected to reaction at 25-50°C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase. In one version of this embodiment, the reaction is carried out at 37°C. In another version of this embodiment, the acidic buffer is a phosphate buffer of pH 5.8 and the levan solution has a levan concentration of 5-15 % (w/v).

In accordance with a thirteenth embodiment of the present invention, there is provided a process of preparing levan fructotransferase, comprising the steps of culturing a bacterial species anchoring a levan fructotransferase gene-carrying, expression plasmid, harvesting and homogenizing the cells, and isolating levan fructotransferase from the cell homogenate. In one version of this embodiment, the levan fructotransferase has histidine residues at its N- or C-terminus and the isolating step is carried out using metal ion-affinity chromatography.

In accordance with an fourteenth embodiment of the present invention, there is provided a process for producing difructose dianhydride IV from sucrose, comprising the steps of reacting a sugar solution at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase to produce levan, purifying the levan from the sugar reaction mixture, partially or completely, reacting a levan solution at 25-50 °C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV, and isolating the difructose dianhydride IV from the levan reaction mixture.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

5 Fig. 1 shows the various steps in a process for the production of difructose dianhydride IV from sucrose.

Fig. 2 shows the restriction map of plasmid pZL8, in which B stand for *Bam*HI, H for *Hind*III, E for *Eco*RV, N for *Nco*I, A for *Ase*I, S for *Sph*I, (B::Sa) for *Bam*HI::*Sau*3A1.

10 Fig. 3 shows a base sequence of the levansucrase gene and an amino acid sequence deduced therefrom.

Fig. 4 shows a SDS-PAGE result of purified levansucrase, in which a standard protein is electrophoresed in lane M, total protein of *E. coli* in lane 1 and the purified levansucrase in lane 2.

15 Fig. 5 shows a SDS-PAGE result of purified levan fructotransferase, in which a standard protein is electrophoresed in lane 1 and the purified levan fructotransferase in lane 2.

Fig. 6 shows a base sequence of the levan fructotransferase gene and an amino acid sequence deduced therefrom.

20 Fig. 7 shows a structure of the recombinant expression vector pUDFA18 carrying the levan fructotransferase gene, in which B stand for *Bam*HI, P for *Pst*I, N for *Nco*I, S for *Sal*I, C for *Cl*aI, and (N/A) for *Nco*I/*A*/III.

Fig. 8 shows the total protein pattern of *E. coli* JUD81 in SDS-PAGE in which a bacterial protein is electrophoresed in lane 1 and a standard protein in
25 lane 2.

Fig. 9 shows the change of the amounts of DFA IV and other

saccharides in the reaction mixture in accordance with the reaction time period.

Fig. 10 is a NMR result for the DFA IV purified.

BEST MODE FOR CARRYING OUT THE INVENTION

5 The present invention pertains to the enzymatic production of DFA IV from sucrose, which is achieved by carrying out the following process steps: a levan production process by levansucrase, a purification and concentration process of levan, a DFA IV production process by levan fructotransferase, and a DFA IV recovery process by crystallization. Below, a description will be given, in
10 detail, of these processes (Fig. 1).

1. Levan Production Process by Levansucrase

In accordance with the present invention, the production of levan is based
15 on the catalysis of the levansucrase isolated from *Z. mobilis* with sucrose serving as a substrate. In the presence of the levansucrase, sucrose is subjected to reaction at room temperature, preferably at 0-15°C and more preferably at 10°C for 20-80 hours and preferably for 20 hours in an acidic buffer solution of pH 3.0-7.0 and preferably of pH 5.0. Therefore, most preferable is to react the
20 sucrose at 10°C for 20 hours in an acetic acid buffer of pH 5.0.

As for the substrate, its examples include refined sugar and raw sugar with preference to refined sugar.

The sugar preferably has a concentration of 10-30 % (w/v) based on the total volume of the culture and most preferably 20 % (w/v).

25 Regardless of whether it is natural or is made by genetic recombination, the levansucrase that is derived from *Zymomonas mobilis* is useful in the present

invention. For the effective catalysis of levan production, the enzyme is used at an amount of 0.42-3.0 U/ml based on the total volume of the reaction solution, and preferably at an amount of 2.08 U/ml.

Usually, gene manipulation leads to the mass production of proteins, which is very advantageous in purification. Thus, the levansucrase of the present invention is preferably obtained through genetic recombination. To this end, first, a plasmid carrying a *Z. mobilis*-derived levansucrase gene, e.g., the plasmid pZL8 obtained from *E. coli* KCTC 8546P, is used as a template to amplify the levansucrase gene by the polymerase chain reaction (PCR) with appropriate synthetic primers. Subsequently, the amplified gene of interest is inserted in a vector which is able to be expressed in a suitable host. For instance, when *E. coli* is used as a host, the levansucrase gene amplified is inserted in pET3d (Stratagene) to construct an *E. coli*-expression plasmid, e.g., pEL11 which is then transformed into the host. This transformed host cell is cultured under an appropriate condition and homogenized, followed by centrifugation. To the supernatant, ammonium sulfate is added to give crude levansucrase.

Since the crude enzyme contains various proteins in addition to levansucrase, a complicated purification procedure is required to obtain pure levansucrase. In order to overcome this problem, there is provided a levansucrase further comprising histidine residues at its N- or C-terminus, which can be simply purified by metal ion-exchange column chromatography, in accordance with the present invention.

Histidine residues can be attached to the N- or C-end of levansucrase as follows. First, PCR is conducted using a set of synthetic primers which are designed to have a histidine base sequence at their 3' or 5'-end while a plasmid carrying a *Z. mobilis*-derived levansucrase gene, e.g., the plasmid pZL8 obtained

from *E. coli* KCTC 8546P is used as a template, so as to yield a large quantity of a levansucrase gene which further comprises a nucleotide sequence coding histidine at its 3' or 5'-end. Thereafter, the amplified gene is inserted in a vector which is expressable in a suitable host. For instance, when *E. coli* is
5 used as a host, the levansucrase gene amplified is inserted in pET3d to construct an *E. coli*-expression plasmid, e.g., pEL11 which is then transformed into the host. This transformed host cell is cultured under an appropriate condition and homogenized, followed by purification with metal ion-exchange column chromatography to obtain pure levansucrase.

10 Experimental data show that, when 0.5-2 U of the levansucrase purified is added in a 20% sugar solution, levan is produced at an amount of 50 g/l after 10 hours of the enzymatic reaction at 10°C.

By using the levansucrase, levan, a water-soluble, homo-polysaccharide, can be mass-produced in a batch process or a continuous process at a high purity
15 ($\geq 98\%$) at a high yield (35-40% from sucrose). In the case of a batch process, because the activity of the levansucrase is inhibited by glucose, a product of the enzyme reaction, the concentration of the substrate must be controlled. Preferable is a 20% substrate solution. As for the continuous process, there is
20 needed a reactor, e.g., a membrane bioreactor, equipped with a means of continuously removing the glucose produced.

After the production of levan, to discard the enzyme used is too extravagant. In this regard, the enzyme may be immobilized to a suitable matrix, such as hydroxyapatite, iron-bead, non-porous glass, wire, etc. in order to reuse the enzyme.

25 In addition to being a substrate for producing DFA IV, the levan produced according to the present invention, as aforementioned, can be used for various purposes, such as a material for foodstuffs and medicines.

2. Purification and Concentration Process of Levan

After completion of the enzymatic production reaction of levan, the
5 resulting levan-containing solution must be deprived of unreacted residual
sucrose, glucose, and oligosaccharides, followed by concentration preferably by
as much as 10-15 %. To this end, available are various techniques, such as
solvent precipitation, ultrafiltration, diafiltration, reverse osmosis,
chromatography, etc. For a small scale, such as a laboratory scale, an organic
10 solvent method using, for example, ethanol is suitable. Only to precipitate
twice or three times in the organic solvent is sufficient to obtain pure levan.
For a large scale, a membrane filtration process is preferably employed, as in
various polymer production processes.

Where the levan is produced in a continuous process, membrane
15 isolation techniques, such as microfiltration (MF), ultrafiltration (UF) or reverse
osmosis (RO), are used to effectively isolate the product of interest or remove
by-products from the reaction solution. These techniques have advantages of
being able to be operated at low temperatures as well as allow the simple
measurement of energy yield and separation capacity. For example, the
20 glucose can be removed from the reaction mixture with the aid of ultrafiltration
(UHF-500-E-90A, 500,000 molecular weight cutoff, A/G Technology Co.) or
MF (CPP-1-k-9A, 0.1 microl, A/G Technology co.). Preferred is UHF-500-E-
90A, which is superior in permeate flux and permeability (permeate flux, 376.47
ml.ft²; permeability, 21.16 ml/min/ft²/psi; 500,000 nominal molecular weight
25 cutoff)

To achieve excellent purification of the levan, the filtration described
above may be used in combination with the organic solvent precipitation.

Alternatively, the purification of the levan may be accomplished by conducting various membrane isolation techniques in multiple steps.

3. DFA IV Production Process by Levan Fructotransferase

5

In accordance with the present invention, DFA IV is produced from partially purified or pure levan by the catalysis of levan fructotransferase.

In the presence of the levan fructotransferase, levan is subjected to reaction at 25-50°C and preferably at 37°C for a time period of 3-10 hours and preferably for 5 hours in a buffer of pH 3.0-7.0 and preferably of pH 5.8. Preferable is a phosphate buffer.

Useful to produce difructose dianhydride is partially or completely purified levan with preference to completely purified levan.

In the reaction solution, the levan is preferably controlled to have a concentration of 5-15 % (w/v) based on the total volume of the culture and most preferably 10 % (w/v).

Regardless of whether it is natural or is made by genetic recombination from transformable microbes such as *E. coli* or yeasts, the levan fructotransferase that is derived from *A. ureafaciens* is useful in the present invention.

The levan fructotransferase of the present invention is preferably obtained through genetic recombination because gene manipulation leads to the mass production of the protein, which is very advantageous in purification. To this end, first, a plasmid carrying an *A. ureafaciens*-derived levan fructotransferase gene, e.g., the plasmid pDA18, is used as a template to amplify the levan fructotransferase gene by PCR with appropriate synthetic primers. Subsequently, the amplified gene of interest is inserted in a vector which is able

to be expressed in a suitable host, such as bacteria or yeast.

In accordance with the present invention, the levan fructotransferase gene amplified is inserted in pUC18 to construct an *E. coli*-expression plasmid pUDFA18, which is then transformed into *E. coli* DH5 α . The new bacterial
5 cell thus obtained was named *E. coli* JUD81 and deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on Sep. 1, 1999 at accession No. KCTC 8961P. The original deposit was converted to a deposit under the Budapest Treaty on Oct. 19, 2000 at accession
10 No. KCTC 0877BP. This transformed host cell is cultured under an appropriate condition and homogenized, followed by centrifugation. Addition of ammonium sulfate to the supernatant allows the production of crude levan fructotransferase.

Since the crude enzyme contains not only levan fructotransferase, but also various other proteins, the crude enzyme must undergo a complicated
15 purification procedure to obtain only levan fructotransferase. In order to overcome this problem, there is provided a levan fructotransferase further comprising histidine residues at its N- or C-terminus, which can be simply purified by metal ion-exchange column chromatography, in accordance with the present invention.

20 Histidine residues can be attached to the N- or C-end of levan fructotransferase as follows. First, PCR is conducted using a set of synthetic primers which are designed to have a histidine base sequence at their 3' or 5'-end while a plasmid carrying an *A. ureafaciens*-derived levan fructotransferase gene, e.g., the plasmid pUDFA18 obtained from *E. coli* KCTC 0877BP is used
25 as a template, so as to yield a large quantity of a levan fructotransferase gene which further comprises a nucleotide sequence coding histidine at its 3' or 5'-end. Thereafter, the amplified gene is inserted in a vector which is expressable

in a suitable host. For instance, when *E. coli* is used as a host, the levan fructotransferase gene amplified is inserted in pUC18 to construct an *E. coli*-expression plasmid, e.g., pUDFA18 with which the host is then transformed. This transformed host cell is cultured under an appropriate condition and
5 homogenized, followed by the purification with metal ion-exchange column chromatography to isolate the levan fructotransferase comprising histidine residues at its N- or C-terminus.

Like levan, DFA IV can be mass-produced in a batch process or a continuous process by using the levan fructotransferase. This enzyme is also
10 immobilized to a suitable matrix, such as hydroxyapatite, iron-bead, non-porous glass, wire, etc. in order to reuse the enzyme.

Experimental data show that, when 20 g of the levan produced by the catalysis of the levansucrase of *Z. mobilis* was allowed to react with a homogenized cell solution containing 25 U of the levan fructotransferase at 37°C
15 in 1000 ml of a phosphate buffer (pH 5.8), DFA IV was produced at a yield of 60 % after 20 hours of the reaction.

4. DFA IV Recovery Process by Crystallization

20 From the levan produced by the catalysis of the levansucrase of *Z. mobilis*, a reaction solution comprising DFA IV 60%, fructose 5%, limited levan 30 %, and other saccharides (mainly, oligosaccharides) 5% is obtained as a result of the decomposition activity of the levan fructotransferase. This reaction solution is subjected to a purification process to isolate DFA IV, or concentrated
25 to some degree to give a solution with a high content of DFA IV. If necessary, the reaction solution itself may be used instead of DFA IV.

Isolation of DFA IV from the resulting reaction solution may be

achieved by conducting crystallization, solvent precipitation, diafiltration, ultrafiltration, reverse osmosis, evaporation, drying, chromatography, etc., alone or in combination. Useful in the present invention is a membrane filtration, which is usually used in various polymer production processes, in practice.

5 In addition to being more stable to heat than sucrose, DFA IV well crystallizes. For the purpose of crystallizing DFA IV, the enzymolyzed solution of levan needs to be concentrated. In this connection, the above-mentioned filtration processes or a popularized standard sugar solution evaporation process may be utilized. Alternatively, the enzymolyzed solution may be primarily
10 concentrated to some degree by filtration and then, heated at 70-80°C until DFA IV reaches a desirable concentration. At this time, a heating temperature exceeding 100 °C causes caramelization. The DFA IV filtrate of the primary filtration may be used as a diluting solution of the levan.

 In a small scale site, such as a laboratory, the DFA IV solution is
15 preferably added with two volumes of an organic solvent, such as ethanol, to precipitate oligosaccharides or limited levan and the resulting supernatant is concentrated using a rotary evaporator, after which the concentrate is controlled to an ethanol concentration of 95 % with 100% ethanol and allowed to stand at 4°C to induce crystal precipitation. Alternatively, while a supersaturated
20 solution of DFA IV is allowed to stand at 4°C to induce natural crystallization, DFA IV crystals are seeded to rapidly form crystals. These batch crystallization processes are, however, disadvantageous in that it is difficult to obtain uniform crystals. In contrast, a continuous process is very useful in crystallizing DFA IV at a uniform size and shape as well as has an advantage of
25 being of low cost compared with the batch process.

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be

construed to limit the present invention.

EXAMPLE

5

EXPERIMENTAL EXAMPLE 1

Cloning of Levansucrase Gene

The isolation of genomic DNA from *Z. mobilis* was executed according to the instruction of Raymond and Tate (Raymond and Tate, Recombinant DNA techniques, p.162, 1983, Addison-Wesley Publ.). After being washed with a TEN buffer (10 mM Tris-Cl pH 7.6, 1 mM EDTA, 10 mM NaCl), 1 g of a mass of *Zymomonas mobilis* ZM1 (ATCC 10988) which had been cultured in a YPS medium (Yeast extract 0.5%, peptone 1%, sucrose 2%) was suspended in 10 ml of an SET buffer (sucrose 20%, 50 mM Tris-Cl pH 7.6, 50 mM EDTA) and treated with lysozyme (Sigma, 5 mg/ml PEN buffer). After 30 min of the enzyme treatment, the mixture was added with 10 ml of a TEN buffer and 1 ml of 10% sodium dodecylsulfate (SDS) and slowly agitated. To the lysed cells was added 2 ml of a 5 M NaCl solution and 20 ml of a TEN buffer and the resulting solution was added with an equal volume of a phenol solution saturated with a TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and agitated for 5 min, followed by centrifugation at 10,000 rpm for 5 min. The supernatant was further centrifuged after being well mixed with chloroform/n-amyl alcohol (24:1). Addition of two volumes of cold ethanol to the resulting supernatant precipitated DNA.

25

After purification, the *Z. mobilis* genomic DNA was partially cut with the restriction enzyme Sau3AI and DNA fragments in a size range of 4-10 kb were isolated with the aid of 1% agarose gel. Separately, pUC119 (Takara, Japan)

was cut with the restriction enzyme *Bam*HI and dephosphorylated. Recombinant plasmids were obtained by ligating the isolated DNA fragments of 4-10 kb to digested pUC119.

Following the techniques of Mandel and Higa (Mandel and Higa, J. Mol. Biol. 53, 159 (1970)) and Inoue et al. (Inoue et al., Gene 96, 23 (1990)), transformation was executed. First, *E. coli* JM109 that had been cultured at 18°C for 36 hours in 30 ml of an SOB medium (Trypton 2%, Yeast extract 0.5%, 10 mM NaCl, 2.5 mM CaCl₂, 10 mM MgCl₂, 10 mM MgSO₄) was kept in ice for 10 min and centrifuged at 2500xg for 10 min, after which the cell pellet was suspended in 10 ml of a TB solution (10 mM PIPES, 55 mM MnCl₂, 250 mM KCl). The resulting suspension was added with dimethyl sulfoxide (DMSO) to the final concentration of 7 %, kept in ice for 10 min, aliquoted at 400 µl in cold tubes, and stored in a liquid nitrogen tank. For transformation, the frozen cell suspension was thawed at room temperature and well mixed with 10 µl of the recombinant DNA (DNA 1 µg) and this mixture was refrigerated for 30 min and subjected to heat-shock at 42°C for 30 sec. Subsequently, the transformation solution was added with 800 µl of an SOC medium (Trypton 2%, Yeast extract 0.5%, 10 mM NaCl, 2.5 mM CaCl₂, 10 mM MgCl₂, 10 mM MgSO₄, 10 mM Glucose), vigorously shaken at 37°C for 1 hour, spread over the surface of an LB agar plate containing ampicillin and chloramphenicol, and cultured at 37°C for 16 hours.

The colonies formed were transferred to an MG agar plate supplemented with sucrose (20 g/l) and ampicillin (50 mg/ml) and cultured at 37°C for 2 days. Spraying GOD-PAP (Böeringer Mannheim) onto the plate allowed the selection of 13 colonies which formed red halos around themselves. Further selection was on the colonies which could be of sucrase activity as well as of levan formativity as measured according to the instruction of Gay et al.

(Gay et al., J. Bacteriol. 153, 1432(1983)). From the *E. coli* colonies, plasmids were isolated and named "pZL8". *E. coli* DH5 α bearing the plasmid was deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on Dec 14, 1993 at deposition No. KCTC 8546P.

5

Gene Identification by Levan Formation

Cultivation of the *E. coli* bearing pZL8 at 37°C for 3 days in an LBS broth (LB broth supplemented with sucrose at 5%) resulted in the detection of polymers similar to those of levan. The culture was centrifuged and the
10 supernatant was added with three volumes of cold ethanol to precipitate polymers. They were dissolved in distilled water and precipitated with ethanol. This ethanol precipitation procedure was carried out twice more. The raw polymers thus obtained were separated by thin layer chromatography (TLC) on silica gel developing with n-butanol/pyridine/water (8/1/1), followed by the
15 coloration with a 1% anisaldehyde-sulfuric acid solution.

Because levan, a macro-molecule with a molecular weight of 5×10^7 Daltons, cannot move on TLC, it was hydrolyzed according to the instruction of Tanaka (Tanaka et al., J. Biochem. 90, 521 (1981)). 100 μ l of the raw polymer solution was boiled for 15 min, along with 50 μ l of 2.5 % oxalic acid, and the
20 hydrolyzed solution was subjected to TLC. The acid hydrolysate was extended for 15 min, only fructose was observed. There were difficulties in analyzing the sugar components with accuracy by TLC because the R_f value was 0.46 for glucose, 0.52 for fructose, and 0.4 for sucrose. To compensate for this, HPLC analysis was executed.

25

20 μ l of each of the hydrolysate samples was filtered through a 0.2 μ m membrane and subjected to HPLC with the aid of an HPLC apparatus, such as sold by Waters, identified as "Model R401", by flowing water at a rate of 0.6

ml/min through a column carbohydrate HPX-87C (Bio-Rad). Detection was conducted using RI (Oven Temp. 85-90°C). All of the samples showed two peaks: a peak was read at 4.19 min for oxalic acid and the other peak at 9.29 min for sucrose. These data, therefore, demonstrated that the polymer obtained
5 above was composed of fructose residues. In addition, no inulin ($\beta(2-1)$ linked polyfructan) formative activity was observed in *Z. mobilis* (Toran et al., *J. Biotechnol. Lett.* 7, 527(1985)). Consequently, it was made certain that the polymers formed in the *E. coli* bacteria bearing the plasmid pZL8 were levan ($\beta(2-6)$ linked polyfructan) (Viikari, L., *CRC Critical Reviews in Biotechnology*
10 7, 237 (1988)) and a levansucrase gene was introduced in the plasmid pZL8.

Gene Identification by Gene Mapping

The recombinant plasmid obtained above was digested with various restriction enzymes and electrophoresed on 1% agarose gel. Based on the
15 DNA fragment data on the agarose gel, a restriction enzyme map of the plasmid pZL8 was drawn, providing the information that the genomic DNA introduced in the plasmid pZL8 was 4.5 kb long.

To be used as a probe, the plasmid pZL8 was labeled with a DNA labeling kit (Böeringer Mannheim). Separately, the *Zymomonas mobilis*
20 genomic DNA prepared above was cut with Restriction enzymes, *EcoRI*, *EcoRV*, *HindIII* and *NcoI* and electrophoresed on 1% agarose gel. Then, the genomic DNA fragments on the agarose gel were transferred to a nylon membrane and hybridized with the probe in accordance with the teaching of Southern (Southern, *J. Mol. Biol.*, 98, 503 (1975)). Referring to the instruction enclosed in the
25 DNA labeling kit, coloration was achieved. Because the plasmid pZL8 has a unique restriction enzyme site for each of *EcoRV*, *HindIII* and *NcoI*, the DNA sequence of 4.5 kb introduced into the plasmid pZL8 must be from *Z. mobilis*.

Gene Identification by Base Sequencing

An examination was made of the levansucrase activity of the mutant bacteria bearing delete pZL8 which was made by use of restriction enzymes. From the examination, it was recognized that the levansucrase gene was positioned in the 1.6 kb base sequence between the *EcoRV* site and the *AseI* site, as shown in Fig. 2.

Delete plasmids, which were constructed from the 1.6 kb DNA fragments with the aid of an *exo/Mungbean* delete kit (Takara, Japan), were amplified. 2 μ l of the prepared plasmids prepared from the mutant cells was denatured with 2 N NaOH, neutralized with ammonium acetate (pH 4.5), allowed to precipitate by ethanol, and dissolved in deionized water. Using a Sequenase kit (USB, U.S.A.), the denatured plasmids were labeled and 4 μ l of the reaction was boiled for 3 min and electrophoresed on 8% polyacrylamide-8M urea gel at 1500-1700 V for 3 hours. The gel was dried and exposed to X-ray film at -70°C for about 8 hours. The reading of the base sequence which appeared on the sensitized X-ray film revealed that the total levansucrase gene including its one termination codon is 1,981 bp long; the structural gene corresponding to the amino acid sequence is 1269 bp long. The base sequence of the levansucrase gene and the amino acid sequence deduced therefrom are as shown in Fig. 3.

Gene Identification by Amino Acid Sequencing

Z. mobilis ZM1 was cultured at 30°C for 24 hours in a YPS medium and the multiplied cells were harvested, suspended in a 20 mM phosphate buffer (pH 6.8), shaken at 30°C for 15 min, and centrifuged according to the introduction of Yanase (Yanase *et al.*, *Biosci. Biotech. Biochem.* 56, 1335 (1992)). The

supernatant was used as a crude enzyme solution.

In this crude solution, ammonium sulfate was saturated up to 50 %, to precipitate proteins which were recovered by centrifugation at 8,000xg for 20 min. The protein mass was dissolved in a 0.02 M phosphate buffer (pH 6.8), followed by dialysis in the same buffer. In this regard, elution was conducted at a rate of 0.5 ml/min through a column (2.5x10 cm) charged with a weak anion exchange resin (DEAE-Toyopearl 650M). In a linear concentration gradient of NaCl from 0 to 0.5 M, the eluate at 0.3 M was collected. The eluate was concentrated and purified followed by Hydroxyapatite column chromatography. After being concentrated, The protein was allowed to precipitate with 20% saturated ammonium sulfate. And finally the concentrate was loaded on a gel filtration column (Superose 12, Pharmacia) to elute a fraction containing a molecular weight of 91,000. The final purification yield was 18.3 fold of the crude enzyme from *Z. mobilis*, with 16.5% of the enzyme recovered in the preparation step (Table 1). The solution was used as a levansucrase solution.

TABLE 2. reductionP Amounts of Levan According to pH of Buffer (g/l)

TABLE 1. Summary of levansucrase purification steps from *Z. mobilis*

Step	Volume (ml)	U total	Protein (mg/ml)	Spec. Act. (U/mg)	Yield (%)	Purifi. Fold
Cell washed	1,300	-a	0.35	-	-	-
1st (NH ₄) ₂ SO ₄	115	-	1.28	-	-	-
Ion-exchange	38	4.35	0.57	0.21	100	1.00
Hydroxyapatite	20	2.58	0.41	0.31	65	1.52
2nd (NH ₄) ₂ SO ₄	2	0.96	0.46	1.04	21	5.07
Superose 12	1.5	0.72	0.13	3.75	16.5	18.3

a: could not be determined.

With the aid of a protein-peptide sequencing system (Applied Biosystems, Model 477A), the amino acid sequence of the purified levansucrase was determined at its N-terminal in the Edman degradation procedure. As a result, a stretch of seven amino acid residues, Met-Leu-Asn-Lys-Ala-Gly-Ile, was sequenced, reflecting the corresponding base sequence of the DNA. In particular, the *levU* gene was revealed to have no base sequences which correspond to the signal peptides, which are usually found in secretory proteins. The nucleotide and amino acid sequences of the levansucrase gene from *Z. mobilis* was registered in the GenBank, U.S.A. (Accession No. AF081588).

10

EXPERIMENTAL EXAMPLE 2

Production of Levansucrase

15 Step 1 Construction of Expression Vector

E. coli KCTC 8546P was cultured at 37°C overnight in an LB medium (yeast extract 0.5 %, trypton 1 %, NaCl 1 %), after which plasmid pZL8 was extracted from the culture, according to the method of Maniatis *et al.* (Maniatis *et al.*, *Molecular Cloning: A Laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., U.S.A.).

20

A levansucrase gene 1296 bp long was amplified by a PCR which employed the following primers 1 and 2 with the plasmid pZL 8 serving as a template:

25 Primer 1: 5'-CGC CGG ATC CAC ATG TTG AAT AAA GCA GGC-3'

Primer 2: 5'-CGC GGA TCC ACA TGT GCA TAA TCA GAA ACG TC-3'

A reaction mixture comprising a 10x Taq polymerase buffer (10 ul), a 10x dNTP mix (10 ul) (dATP, dCTP, dGTP, and dTTP, 2 mM each), the primers 1 and 2 (1 ul (100 pM), each), the plasmid pZL8 DNA (5 ul (20 ng)), and deionized water (72 ul) was thermally treated, along with a mineral oil (100 ul), at 95°C for 5 min to denature the DNA. The reaction mixture was added with a Taq polymerase (1 ul (5 U)) after being cooled to 72°C and then, allowed to undergo 25 thermal cycles in which heating processes were performed in the order of at 95 °C for 1 min, at 55°C for 2 min and at 72°C for 3 min.

The PCR product thus obtained was digested with the restriction enzyme *Afl*III and introduced into the *Nco*I site of the plasmid pET3d (Stratagene) to construct the *E. coli*-expression plasmid pEL11.

Step 2 Transformation of *E. coli*

E. coli DH5 α and BL21 (DE3) were separately cultured at 18°C for 36 hours in 30 ml of an SOB medium (Trypton 2%, Yeast extract 0.5%, NaCl 10 mM, calcium chloride 2.5 mM, magnesium chloride 10 mM, magnesium sulfate 10 mM), followed by centrifugation to harvest the bacteria. Subsequently, the cultured bacteria were separately suspended in 10 ml of a TB buffer (Pipes 10 mM, manganese chloride 55 mM, calcium chloride 15 mM, potassium chloride 250 mM), followed by centrifugation to collect the cells. The cell mass was resuspended in 12 ml of a TB buffer and after being added with DMSO (dimethyl sulfoxide) at a concentration of 7 %, the suspension was kept on ice and stored at an aliquot of 400 ul in a liquid nitrogen tank.

After being thawed, the *E. coli* DH5 α was added with 10 ul of the plasmid pEL11 constructed in the Step 1 and kept on ice for 30 min, followed by heat shock at 42°C for 30 sec for transformation. The transformed cell mixture was added with 800 ul of an SOC medium, vigorously agitated at 37°C for 60

min, and spread on an LB agar containing 170 mg/ml of ampicillin, which was then incubated at 37°C for 16 hours. Colonies formed on the agar medium were taken and cultured in LB broths containing ampicillin. Plasmids were extracted from the cells cultured and tested for whether they carried the
5 levansucrase gene, by restriction enzyme mapping.

E. coli BL21 (DE3) was transformed by the recombinant plasmid which had been ascertained to carry the levansucrase gene, spread on an ampicillin-added LB agar, and incubated at 37°C.

10 Step 3 Production and Isolation of Levansucrase

The transformed cells were inoculated in M9-ZB media (Trypton 10g, NaCl 5g, NH₄Cl 1g, KH₂PO₄ 3g, Na₂HPO₄ 6g, Glucose 4g, 1M MgSO₄ 1 ml) and cultured with agitating. When the absorbance at 600 nm of the culture reached 0.7 during the cultivation, IPTG (Sigma) was added to the concentration
15 of 1 mM with the aim of inducing the expression of the levansucrase.

To the cell pellet which was obtained by centrifuging the culture, a 100 mM Tris buffer (pH 7.0) was added at an amount of 1/10 volume of the culture, and the suspended cells were homogenated by sonicating three times for 30 sec with the aid of a sonifier (Branson), followed by centrifugation at 12000xg for
20 60 min. The supernatant thus obtained was added with ammonium sulfate to the concentration of 20 % and centrifuged to give above 94 %-pure levansucrase. This crude enzyme mass was found to have a levansucrase activity of 7.8 U/ml, if harvested at 4 hours after the IPTG induction, as measured according to the instruction described in Song et al. (song and Rhee,
25 *Biotechnol. Lett.* 16, 1305 (1994)) Example 2. It amounted to 30 % of the total quantity of the proteins produced from the *E. coli*.

COMPARATIVE EXAMPLE 1

Z. mobilis ATCC 10988 was cultured in a YPS medium (Yeast extract 1%, potassium phosphate 0.1%, sucrose 20%) at 30 °C for 18 hours, and from
5 the culture was obtained levansucrase which was measured to have an activity of 1.5 U/ml.

Therefore, the amount of the levansucrase produced from the transformed cells in Experimental Example 2 was 5.2 times as much as that of the levansucrase produced from *Z. mobilis* ATCC 10988.

10

EXAMPLE 1

While *E. coli* was cultured as in the Step 3 of Experimental Example 2, the culture was taken out at 10 ml at regular intervals of time, and centrifuged.
15 To the cell pellet was added a 100 mM Tris buffer (pH 7.0) at an amount of 1/10 volume of the culture, and the suspended cells were homogenated by sonicating three times for 30 sec with the aid of a sonifier, followed by centrifugation at 12000xg for 60 min. The supernatants were electrophoresed on a 10% polyacrylamide gel.

20 The polyacrylamide gel electrophoresis showed that the levansucrase was water-soluble by 6 hours after the IPTG induction and was gradually converted into a water-insoluble one from 8 hours after the IPTG induction with a gradual decrease in enzyme activity. At 10 hours after the IPTG induction, the levansucrase was measured to have an enzyme activity of 10 %.

25

EXAMPLE 2

A PCR was carried out using Primer 1 and Primer 3 (5'-CGA CAT GTT AGT GAT GGT GAT GGT GAT GTA AAG ACA GGG CTG-3') while the plasmid pZL8 isolated from *E. coli* KCTC 8546P served as a template. Using the PCR product, the same procedure as Experimental Example 2 was repeated to construct the plasmid pEL12 capable of expressing the levansucrase carrying histidine residues at its C-terminus in *E. coli*, which was then used for the transformation of *E. coli* BL21 (DE3).

The transformed *E. coli* BL21 (DE3)/pEL12 was deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on April 25, 1995 at deposition No. KCTC 8861P.

EXAMPLE 3

Ni-NTA resin (Quagen, U.S.A.) was charged in a 1.5x20 cm column through which a 50 mM phosphate buffer (pH 8.0) was then flowed to give a column for metal ion-affinitive chromatography.

E. coli KCTC 8861P of Example 2 was cultured in the same manner as the Step 3 of Example 1 and sonicated. The cell homogenate was loaded on the column which was then washed with a buffer (50mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.0), followed by detaching the enzyme from the resin by flowing a 0.3 N imidazole solution (pH 7.0). The result is given in Fig. 4. As shown in Fig. 4, the enzyme was purified with above 95% of homogeneity (lane 2), from the total protein of *E. coli* lysate(lane 1).

EXAMPLE 4

Using the levansucrase carrying histidine residues at its C-terminal,

obtained in Example 3, the experimental procedures of Examples 3 to 8 were repeated. The data demonstrate that the recombinant levansucrase to the C-end of which histidine residues were attached, has levan production ability almost identical to that of natural levansucrase.

5

EXPERIMENTAL EXAMPLE 3

Levan Production

10 In 5 ml of 50 mM acetic acid buffers which were controlled to pH 3-7.5, sucrose was dissolved to the concentration of 10 %, followed by the addition of 0.42 U of the crude enzyme obtained in Experimental Example 2. The resulting mixture was allowed to react at 10°C for 5 hours and 48 hours, after which a measurement was made of the amount of the levan produced.

15 The results are given in Table 2, below. As apparent from the data, levan was produced in the largest quantity when the buffer was pH 5.

TABLE 2. Production Amounts of Levan According to pH of Buffer (g/l)

Temp.	PH									
	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5
5	6.6	8.0	9.3	10.0	10.5	9.2	7.0	5.5	4.3	3.0
48	26.7	30.0	32.9	35.2	35.9	31.1	28.0	27.5	22.1	17.1

20

EXAMPLE 5

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 10 % and was added 1.05 U of the crude enzyme obtained in Experimental Example 2. The resulting mixture was allowed to react at –

3°C, 0°C, 5°C, and 10°C for up to 200 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

The results are given in Table 3, below. As apparent from the data, a reaction temperature of 10°C was the best condition for the crude enzyme to produce levan until 50 hours of the reaction.

TABLE 3. Production Amounts of Levan According to Reaction Temp. (g/l)

Temp.	Time period					
	4	10	27	46	78	167
-3°C	7.3	10.5	20.5	30.0	34.5	30.5
0°C	10.1	15.7	29.5	36.5	37.2	32.3
5°C	11.3	22.2	33.2	36.4	38.4	33.0
10°C	15.2	29.3	34.9	36.7	37.5	29.7

10

EXAMPLE 6

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentrations of 5-40 % and was added 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixture was allowed to react at 10°C for up to 60 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

The results are given in Table 4, below. As apparent from the data, the levan amounts produced in a sucrose concentration of 30 % were greater than in any other sucrose concentration.

TABLE 4. Production Amounts of Levan According to Sucrose Concentration (g/l)

Sucrose Concentration	Time period					
	2	11	18	25	36	59
5	12.1	19.0	21.8	21.6	21.4	20.8
10	14.8	38.2	36.7	39.1	34.2	34.9
20	14.1	51.4	56.7	52.9	51.4	52.0
30	14.6	52.4	71.1	66.9	69.8	59.4
40	11.5	39.1	59.8	58.8	60.0	56.4

5

EXAMPLE 7

In 5 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 10 % and were added 0.42U, 1.05 U, and 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to 150 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

The results are given in Table 5, below. It is apparent from the data that the levan is produced at larger amounts by the greater activity of the enzyme.

15

TABLE 5. Production Amounts of Levan According to Enzyme Concentration (g/l)

Enzyme Concentration	Time period				
	4	12	48	96	144
0.42U	2.5	4.0	21.7	35.7	39.0
10.5U	4.9	12.9	34.9	48.5	42.2
20.8U	24.9	44.9	49.0	49.2	46.2

EXAMPLE 8

In 5 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 20 % and were added 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to 36 hours, after which a measurement was made of the amounts of the levan and other saccharides produced according to the condition parameter.

The results are given in Table 6, below. It is apparent from the data that the levan is produced at larger amounts by the greater activity of the enzyme.

TABLE 6. Production Amounts of Levan According to Reaction Time (g/l)

Saccharides	Time period					
	0	6	11	18	25	36
Levan	0	28.3	51.4	56.7	52.9	51.4
Residual Sucrose	200	161	125	120	98	10
Glucose	0	237	36.0	50.4	74.6	104.8
Fructose	0	2.5	5.8	6.2	6.5	7.2

EXAMPLE 9

15

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved raw sugar (DaeHan Sugar Co.) to the concentration of 20 % and were added 1.05 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to about 91 hours, after which a measurement was made of the amounts of the levan and other saccharides produced. Levan was produced at an amount of 12.5 g/l after 11 hours of the reaction, 25.0 g/l after 25 hours, and 45.8 g/l after 91 hours.

EXPERIMENTAL EXAMPLE 4

Selection of Strains Containing Levan Fructotransferase Capable of Producing5 DFA IV from Levan

Strains were selected from samples taken from soil in Korea. 0.5 grams of each of the soil samples was suspended in 9.5 ml of distilled water and the suspensions were successively diluted to 10-fold, 100-fold and 1000-fold. 200 ul of each of the dilutions was spread on the surface of a selective agar plate, and incubated at 30°C for 72 hours in an incubator. The selective agar medium contained 0.5 % (w/v) of levan as a major carbon source, along with 0.3 % of NaNO₃, 0.05 % of MgSO₄, 0.02 % of MnCl₂, 0.1 % of K₂HPO₄ and 1.5 agar/L. A solution of a predetermined amount of levan in water was passed through a 0.45 micron filter and the filtrate was mixed with the other nutrients before being added to a sterilized medium. After the incubation, colonies around which halos had been formed were primarily selected as being capable of decomposing levan.

Inocula picked from the primarily selected colonies were inoculated in selective broths, each containing levan as a major carbon source, and cultured at 30°C for 72 hours in an incubator. Investigation was made into the growth of the strains inoculated and the metabolites resulting from the metabolism of levan through thin layer chromatography (TLC). Of the 250 strains primarily selected, 8 strains of microbes were found to produce difructose dianhydride. Finally, one strain which stably produced difructose from levan with high enzyme activity was selected and named "K2032".

Identification of Strain K2032

The identification of the selected strain K2032 was achieved by examining its biochemical characteristics and the composition and content of its fatty acids and quinones and comparing them with the database previously accumulated. Many experiments revealed that strain K2032 is Gram-variable and is able to utilize glucose, fructose, galactose, arabinose, mannitol, xylose, ribose, sorbitol, cellobiose, glycogen, sucrose, acetic acid, propionic acid, and salicin, but not rhamnose, malonate, adipic acid, N-acetyl glutamic acid, and adipic acid. As for its intracellular fatty acid composition, the bacteria comprises C15 anteiso/C17 anteiso/C16 iso at a ratio of 51%/14.5%/3.6% with C16/C14 being at a ratio of 17.8%/7.1%. As a result of the analysis of quinones, which are important components for the chemotaxonomy of microbes, the bacteria contained menaquinone MK-9(H₂).

With the analysis results, the strain K2032 was identified as *Arthrobacter ureafaciens*, and thus, named *A. ureafaciens* K2032 (Table 7).

TABLE 7. Phenotypic and chemotaxonomic characteristics of strain K2032

	Gram reaction	variable (+ when young)
20	Acid from glucose	-
	Nitrate reduction	-
	Starch hydrolysis	-
	Growth in 10% NaCl	-
	Utilization of D-glucose, D-fructose, D-galactose, L-arabinose, D-mannitol, D-xylose, D-ribose	+
25	D-sorbitol, cellobiose, glycogen, sucrose, acetic acid, propionic acid, salicin	
	Utilization of L-rhamnose, malonate, adipic acid, N-acetyl glutamic acid, L-serine	-
30	Cellular fatty acid;	
	C15 anteiso/C17 anteiso/C16 iso	51%/ 14.5%/ 3.6%
	C16/C14	17.8%/ 7.1%
	Major menaquinone	MK-9(H ₂)

EXPERIMENTAL EXAMPLE 5

Purification of Levan Fructotransferase from *Arthrobacter*

5 *A. ureafaciens* K2032 was inoculated in a selective broth containing levan as a major carbon source and cultured at 30°C for 72 hours in an incubator. Investigation into the growth and levan fructotransferase activity of the bacteria provided the knowledge that the largest enzyme activity is obtained after 10 hours of the incubation. From the culture incubated for 10 hours, 10 the enzyme was purified (Fig. 5). In this regard, the purification was achieved by successively using acetone precipitation, ion exchange chromatography (DEAE 650-M and Mono Q), and gel filtration chromatography, resulting in producing levan fructotransferase with a specific activity of 2269 U/mg protein at an amount of 1.1 mg at a yield of 15 29.8 % (Table 8).

TABLE 8. Summary of levan fructotransferase purification from *A. ureafaciens* K2032.

Purification step	Volume (ml)	Total activity (U)	Protein (mg)	Spec. Act. (U mg ⁻¹ protein)	Purification Fold	Yield (%)
Culture supernatant	3,800	8,360	182.3	45.9	1.0	100
Acetone precipitation	700	5,670	71.3	79.5	1.7	67.8
Ion-exchange adsorption	5.5	4,821	5.0	964.2	21.1	57.7
2 nd Ion-exchange adsorption	3.2	3,416	2.6	1313.8	29.2	40.9
Gel permeation (Superose 12)	1.2	2,496	1.1	2269.0	49.8	29.8

Characterization of the Purified Enzyme

The purified enzyme was 51,000 in molecular weight as measured by SDS-PAGE while being measured to have a molecular weight of 96,000 by gel filtration chromatography. Thus, it was identified as being a dimer in aqueous conditions. An amino acid analysis showed that the enzyme had at its C-terminus a stretch of the amino acid sequence SAPGSLRAVYHMTPPSGXLXDOQ wherein X residues are not identified. Maintaining its stability in a pH range of 4-10.5, the enzyme showed optimal activity at around pH 6.0. Also, it was of optimal activity at 55 °C. After being allowed to stand at 50°C for 30 min, the enzyme was observed to have a remnant activity of 90 % or greater (Table 9). Whereas being inhibited by Mn^{2+} , Fe^{2+} and Hg^{2+} , the activity of the enzyme was enhanced by Na^{2+} and Ca^{2+} .

TABLE 9. Effect of additives on the activity of levan fructotransferase from *A. ureafaciens* K2032

Additive	Concentration (mM)	Relative activity (%)
Control	-	100
NaCl	1, 10	125, 142
NH ₄ Cl	1	46
MnCl ₂	1	9
CaCl ₂	1, 10	156, 181
NiCl	1	74
ZnCl ₂	1	23
LiCl	1	92
MgCl ₂	1	63
FeCl ₂	1	4
HgCl ₂	1	0
KCl	1	89
EDTA	1, 10	95, 94
SDS	1, 10	101, 32

EXPERIMENTAL EXAMPLE 6

Isolation of Genomic DNA from *Arthrobacter*

Isolation of a gene coding for a levan fructotransferase from *A. ureafaciens* was performed using a genome DNA isolation kit, such as that sold by Bio 101, identified as "G NOME™ DNA ISOLATION KIT", according to its instruction. First, *A. ureafaciens* K2032 was inoculated in 50 ml of a selective broth and cultured for 24 hours, followed by centrifugation at 3,000 rpm for 5 min to harvest cells. To the cell pellet was added the cell suspension solution (10 mM Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0)) to the volume of 1.85 ml. This cell suspension was well mixed with 100 ul of the cell lysis/denaturing solution (0.5% SDS), along with 50 ul of the RNase solution, and incubated at 55 °C for 15 min. To remove proteins from the sample, 25 ul of the protease was added and incubated at 55°C for 1 hour. After 500 ul of the salt out solution was added, the mixture was aliquoted in 1.5 ml tubes which were then cooled at 4°C for 10 min. Following centrifugation at 12000 rpm for 10 min, the supernatant was transferred to a 15 ml tube, in which 2 ml of TE buffer and 8 ml of 100 % ethanol were added to induce DNA precipitation. The DNA precipitates were collected by centrifugation at 12,000 rpm for 15 min, dried in the air and dissolved in a TE buffer.

Cloning of Levan Fructotransferase-Coding Gene

The genome DNA prepared from *A. ureafaciens* was digested with various restriction enzymes and electrophoresed on 1% agarose gel, after which DNA fragments in a size range of 3-4 kb were eluted from the agarose gel. The partial genomic DNA fragments were ligated into the cloning vector pBluescript KSII⁺ which was previously cut with *Bam*HI. This recombinant

plasmid was transformed into *E. coli* DH5 α . Of the transformants, the cells carrying a gene coding for a levan fructotransferase were selected as follows.

First, the -V-Y-H-M-T-P- in the N-terminal amino acid sequence of the K2032 strain and the amino acid sequence (-E-C-P-D-L-F-, p2 primer), which
5 was found to be in a homology relationship with levan fructotransferase, were used to synthesize degenerated primers (p1: 5'-GTNTAYCAYATGACNCC and p2: 5'-AABAYYTCSGGYCARTC). Using these primers, a standard PCR was carried out with the genomic DNA of K2032 bacteria serving as a template. The PCR products ranging, in size, from 600 to 650 bp were eluted from the gel
10 and introduced into shuttle vectors for *E. coli*. As a result of restriction enzyme gene mapping, the DNA fragments introduced were identified as being divided into four kinds and the vectors carrying these DNA fragments were called pDA11, 17, 18 and c8, respectively. In addition, base sequencing analysis of the four DNA fragments showed that the DNA fragment inserted in pDA11 and
15 the levan fructotransferase gene of *A. nicotinovorans* are in high homology (85 %) relationship. Therefore, the DNA fragment introduced in pDA18 could be used as a probe for the Southern hybridization with the DNA prepared from *A. ureafaciens* K2032 with the aim of detecting the levan fructotransferase gene anchored in the bacteria. Before the Southern hybridization, the genomic DNA
20 of *A. ureafaciens* K2032 was digested with various restriction enzymes. As a result of the Southern hybridization, a signal was detected in a 5.6 kb DNA fragment upon digestion with *Cla*I, in a 8.0 kb DNA fragment upon digestion with *Pst*I, and a DNA fragment longer than 10 kb upon digestion with *Bam*HI. Accordingly, the genomic DNA was cut with either *Cla*I or *Pst*I and
25 electrophoresed on a 1% agarose gel, after which DNA fragments in a size range of 5-10 kb were eluted from the agarose gel. The partial genomic DNA fragments were ligated into the cloning vector pBluescript KSII⁺ which was

previously cut with *ClaI* or *PstI*. These recombinant plasmids were transformed into *E. coli* DH5 α . Of the transformants, the cells carrying a gene coding for a levan fructotransferase were selected by Southern hybridization. Finally, when cutting with *ClaI*, there was obtained plasmid pD*Cla* carrying a
5 5.6 kb DNA fragment. On the other hand, when cutting with *PstI*, there was obtained plasmid pD*pst* carrying a 8.0 kb DNA fragment.

Isolation, Analysis and Subcloning of the Recombinant Plasmids

DNA preparation from the transformed *E. coli* was carried out by an
10 ordinary boiling method and with the aid of a commercially available kit. To analyze the recombinant plasmids, they were digested with restriction enzymes and electrophoresed on 1% agarose gel.

The restriction enzyme gene mapping of the DNA fragments introduced in pD*Cla* and pD*pst*, the analysis of gene loci, and the Southern blotting with
15 DNA fragments showed that pD*Cla* and pD*pst* carried an N-terminal half and a C-terminal half of the gene of interest with an overlapping stretch of about 200 bp therebetween. No enzymatic activity was detected from the bacteria which anchored the two plasmids respectively. According to a base sequencing analysis, the sites for restriction enzymes *PstI*, *ApaI* and *NotI* were found to be
20 present in both of the plasmids. By taking advantage of these common restriction enzyme sites, there was constructed plasmid pDF8 which has a complete open reading frame (ORF). A DNA fragment 3.5 kb long was introduced in the plasmid pDF8 and enzymatic activity was detected from the bacteria anchoring this plasmid.

25

Base Sequence of the Cloned Levan Fructotransferase Gene and Its Putative Amino Acid Sequence

In order to analyze the nucleic acid base sequence of the 3.5 kb DNA introduced into pDF8, DNA ladder fragments which showed a regular distance of about 300-400 bp were made and subjected to a sequencing process. As a result, a sequence of 3345 bases was ascertained and found to have only one ORF. Using the BLAST search, which is available from the Internet, the ORF was investigated for homology. In result, a levan fructotransferase gene was detected as being high in the homology with the ORF, and thus, it was named *lftA*. Fig. 6 shows the base sequence of *lftA* and the amino acid sequence deduced therefrom.

The newly isolated levan fructotransferase structural gene is composed of 1566 bp which corresponds to 521 aa. The gene of the present invention has a signal sequence consisting of 99 bp (corresponding to 33 aa) at its N-terminus and therefore, the putative completed protein may be about 56 kDa in molecular weight. Its isoelectric point was found to be pH 5.15. This levan fructotransferase gene is longer by 15 bases (5 amino acids) than is the levan fructotransferase gene derived from *A. nicotinovorans* GS-9. These two genes are 81% in the homology of amino acid sequence (76% in the homology of base sequence). Consequently, the levan fructotransferase gene of *A. ureafaciens* K2032 was identified as being new and its base sequence was registered in the GenBank, U.S.A. (Accession No. AF181254).

EXPERIMENTAL EXAMPLE 7

Development of Highly Expressable, Transformed E. coli Carrying Recombinant Levan Fructotransferase Gene

Using the restriction enzyme *Pst*I, the *lftA* gene was cut at its signal sequence, followed by Klenow treatment to give a blunt end. The *lftA* gene

was divided by cutting with *NorI*. The Klenow-treated DNA fragment was further digested with *SmaI* and ligated to the pUC118 vector which was previously treated with CIP, so as to construct pUDFA18. This was introduced into *E. coli* DH5 α . Fig. 7 shows the plasmid pUDFA18 which is obtained by inserting the *lftA* gene in the expression vector pUC118. Fig. 8 shows the total protein pattern of *E. coli* JUD81 in SDS-PAGE. The *E. coli* JUD81 is the *E. coli* DH5 transformed with pUDFA18.

EXPERIMENTAL EXAMPLE 8

10

Production of DFA IV from Levan by Use of Recombinant Levan Fructotransferase

In 1,000 ml of warm water was dissolved 20 g of levan, cooled and controlled to pH 6.5 with a phosphate buffer. To the resulting solution, *E. coli* JUD81 homogenate was added and allowed to react at 37°C. Fig. 9 shows the change of the amounts of DFA IV and other saccharides in the reaction mixture in accordance with reaction time period. After 40 hours of the reaction, the reaction mixture was allowed to stand in hot water for 5 min to inactivate the remaining enzyme and loaded onto a charcoal column (diameter 6.5 cm, height 20 cm). The column was washed with distilled water until no saccharides were detected in the washings. The column was again washed with 1,000 ml of 5 % ethanol and then with 1,000 ml of 25 % ethanol to elute the absorbed DFA IV. The effluents containing DFA IV were collected and concentrated to a volume of 30 ml by use of a rotary evaporator. To the concentrate, 100 % ethanol was added to the final ethanol concentration of 95 % or higher, so as to crystallize DFA IV. The DFA IV precipitates were washed many times with pure ethanol

and dried to yield 2.5 g of pure DFA IV.

INDUSTRIAL APPLICABILITY

5 The analysis data from NMR, HPLC and TLC after acidolysis demonstrate that the DFA IV obtained is identical to a standard sample. Fig. 10 is a NMR result for the DFA IV obtained.

10 The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

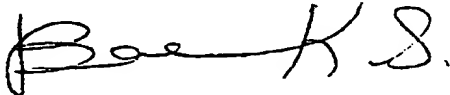
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : SONG, Ki Bang
Hanbit Apt. 110-802, #99, Oun-dong, Yusong-ku, Taejon 305-333,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> JUD81	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0877BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on September 01 1999 and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on October 16 2000	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: October 19 2000

CLAIMS:

1. A novel microorganism *A. ureafaciens* K2032, which shows an activity of selectively producing difructose dianhydride IV from levan and an activity of degrading levan.

5

2. A novel levan fructotransferase of the following amino acid sequence 1, derived from *A. ureafaciens* K2032, which can hydrolyze levan to selectively produce difructose dianhydride IV:

10 1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V
91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A
121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T
15 151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N
181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F
211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L
241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A
271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A
20 301 V T W F S I D A P E T K R L A I A W M N N W K Y A A R D V P
331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L
361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W
391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T
421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S
25 451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
511 G I V V R E I G Q A I *

3. A novel levan fructotransferase gene, which has a base sequence
30 coding for the amino acid sequence of claim 2.

5. A novel levan fructotransferase gene as set forth in claim 3, wherein the base sequence is the following sequence 3:

30

1 GCGGTGCACCCGACTTCCCTCGACGACCACCGTCCCCCTACCGGCCGACCGCCGGCCGACTGCTCCTCAGCCTAGACGGGCCCTC

AvaI

AvaI

91 CTGAGGCTCTTCGTGGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGCCGGCGGTGTACCGCGAGCCTCGAGACGGCA

181 CGGCCAGGAACCTGTCACGTGACCGCATGACGTCGAGGCGCCACGCGATGCTGACGCCCTGAACCTGCCGCCGTTCTGGGCTGACGA

271 GCGCTCCACCCCGACAGCTCTCCTTCTACCGCTGCCCGAACCCAGGGTGGACGCTTCGTGCGGCCACCCGTCACGAGAGGAACAGCA
Pal
361 ATGACGCGCGGCATCTCACGCCGCGCGTGTCCAGGGAGCGCGCGCGGAGCACTCGCCCTGATCTTCGGCGGTGCTGTGCCGCTGCA
1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
5 451 GCGCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGTCAAC
31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
541 ACCCAGCGCGCTACCACTGTACTACCTGCACTCCGACCAGAACACGCGCCCGCGGCTGGGACCACGCGAGACGACCGGCGCTC
61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V
631 GCCTTCACGACACCGGACCGTGTATGCCGTGCGGCCGACTTCCCGTGTGGTCCGGGTGCGCGGTCTGCGACCGCGAACACGGCA
10 91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A
721 GGGTTCGGCGCGCGCGGTGCTGCGCTCGCGACCCAGCCGACCGGCGTCCGCAAGTACCAAGAGCAGTACCTCTACTGGTCGACC
121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T
811 GACGGCGGGTTCAGTTCACCGCCCTGCCGACCCCGTATCGTCAACACCGACGGTCGCGCCGCCACCGCCCGCGAGATCGAGAAC
151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N
15 901 GCCGAGTGGTTCGCGACCCCAAGATCCACTGGGACACCGCCCGCGAGAATGGGTCTGCGTCACTCGGACGACTGCGGTACGCCGCTTC
181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F
991 TACACCTCGCCGAACCTGCGGACTGGACACTTCGCGCAACTTCGACTACCCGAACCAACGCGCTCGGCGGCATCGAGTGCCCCGACCTG
211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L
1081 TTCGAGATACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCGCTACGGCATCGGCTCCCCATGACGTACGCC
20 241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A
1171 TACTGGACAGGCACCTGGGACGGCGAGCAGTTCACGCCGACGACCTCACCCCGCAATGGCTCGACTGGGGCTGGGACTGGTACCGGGC
271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A
A
ClnI AclI
1261 GTCACCTGGCCATCGATCGACGCGCCGAGACCAAGCGCTCGCCATCGCGTGGATGAACAACCTGGAAGTACGCGCGACGCGACGTCCCC
25 301 V T W P S I D A P E T K R L A I A W M N N W K Y A A R D V P
1351 ACCGACGCATCCGACGGCTACAACGGGAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCGACAGCCTGGCGGCTGGTACCCCTCTCG
331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L
NcoI
1441 AGCACCCCGTGGCAGCGCTGACGAACTACGTACCCGCCACCACTACCTCCCGACCGGACCGTTCGACGGCAGCGCGCTCTGCCATGG
30 361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W
1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCTGGGACACCGCGAGAACGTCGGCATCTCGGTGGGCGGCTCCCCGACGGAACC
391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T
1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGAGGACCTCCGACCTCGCGGGTACTCGCTCGCCCCCTACTCG
421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S
35 1711 CGAGCCGCCGCCCATCGACCCCGGCGCGGATCCGTGCACCTGCGCATCTCGTCGACACCCAGAGCGTGCAGGTCTTCGTCAACGCC

451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
 1801 GGCCACACCGTGCTCTCCAGCAGGTCCACTTCGCCGAGGGCGACACGGGAATCTCGTCTACACCGACGGCGGCCCGCACACTTCACC
 481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
 SmaI
 5 1891 GGCATCGTCGTCGCCGAGATTGGCCAGGCGATCTAGGCGATGCACACCACACCGCTCACCGAAGCCGCGCCCCGGGAGACGACGGCCGAC
 511 G I V V R E I G Q A I *
 1981 AATCGACACGTCCTCGTCGTT

6. A recombinant expression vector, carrying the levan fructotransferase
 10 gene of claim 3.

7. A recombinant expression vector as set forth in claim 6, wherein said
 expression vector is pUDFA81.

8. A novel transformant transformed with a recombinant expression
 15 vector as set forth in claim 6.

9. A novel organism *E. coli* JUD81 (KCTC 0877BP), which is prepared
 by transforming *E. coli* DH5 α with the expression vector pUDFA81 of claim 7.

20

10. A process for producing difructose dianhydride IV from levan, in
 which a levan solution is subjected to reaction at 25-50°C for 3-10 hours in an
 acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase.

11. The process as set forth in claim 10, wherein the reaction is carried
 25 out at 37°C.

12. The process as set forth in claim 10, wherein the acidic buffer is a

phosphate buffer of pH 5.8

13. The process as set forth in claim 10, wherein the levan solution has a levan concentration of 5-15 % (w/v).

5

14. The process as set forth in claim 10, wherein the levan fructotransferase has histidine residues at its C- or N-terminus.

15. A process of preparing levan fructotransferase, comprising the steps
10 of:

culturing a bacterial species anchoring a levan fructotransferase gene-carrying, expression plasmid;

harvesting and homogenizing the cells; and

isolating levan fructotransferase from the cell homogenate.

15

16. The process as set forth in claim 15, wherein the levan fructotransferase has histidine residues at its N- or C-terminus.

17. The process as set forth in claim 15, wherein the isolating step is
20 carried out using metal ion-affinity chromatography.

18. A process for producing difructose dianhydride IV from sucrose, comprising the steps of:

25 reacting a sugar solution at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase to produce levan;

purifying the levan from the sugar reaction mixture, partially or completely;

reacting a levan solution at 25-50°C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV; and

isolating the difructose dianhydride IV from the levan reaction mixture.

5

19. The process as set forth in claim 18, wherein the levansucrase is derived from *Z. mobilis*.

20. The process as set forth in claim 18, wherein the sugar solution is
10 reacted at 0-15°C.

21. The process as set forth in claim 18, wherein the sugar solution has a sugar concentration of 10-30 % (w/v).

15 22. The process as set forth in claim 18, wherein the levan solution is reacted at 37°C.

23. The process as set forth in claim 18, wherein the acidic buffer for the sugar solution is an acetic acid buffer of pH 5.0 and the acidic buffer for the
20 levan solution is a phosphate buffer of pH 5.8

24. The process as set forth in claim 18, wherein the levan solution has a levan concentration of 5-15 % (w/v).

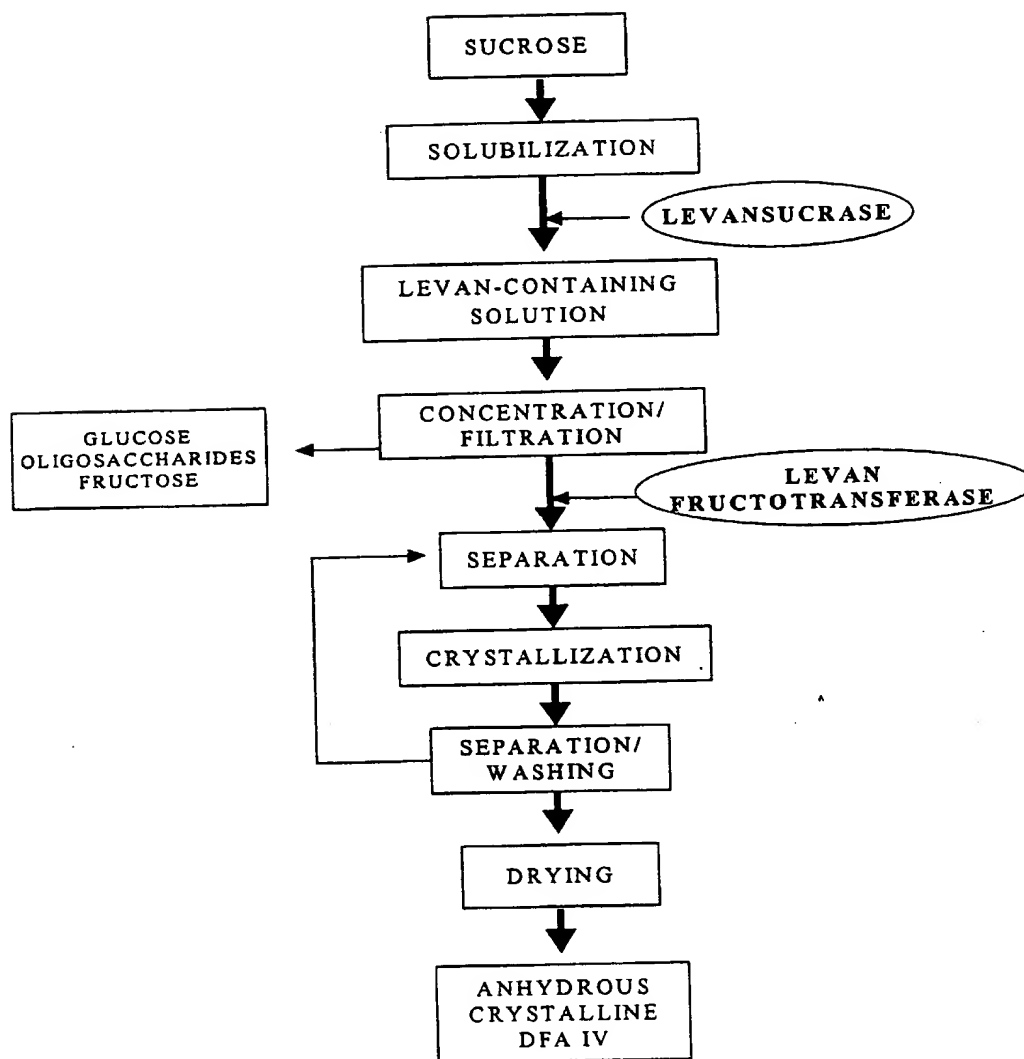
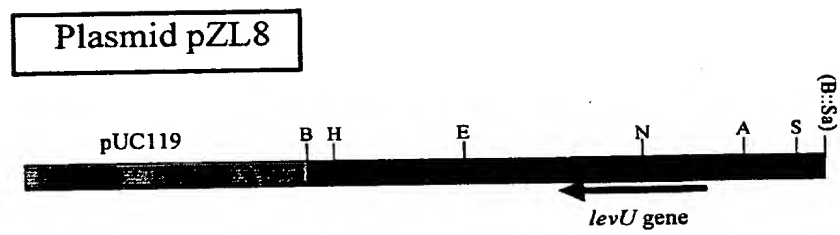
Fig. 1

Fig. 2

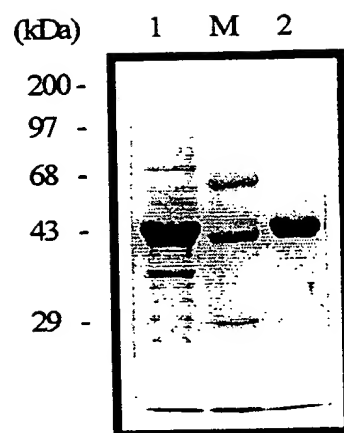


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Fig. 3

[illegible]

Fig. 4



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Fig. 5

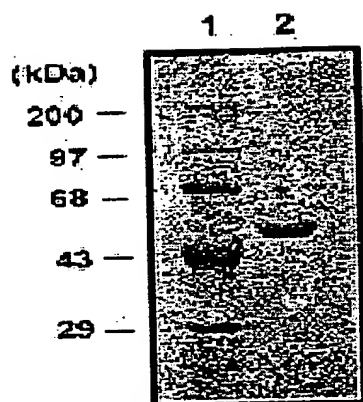


Fig. 6

[illegible]

Fig. 7

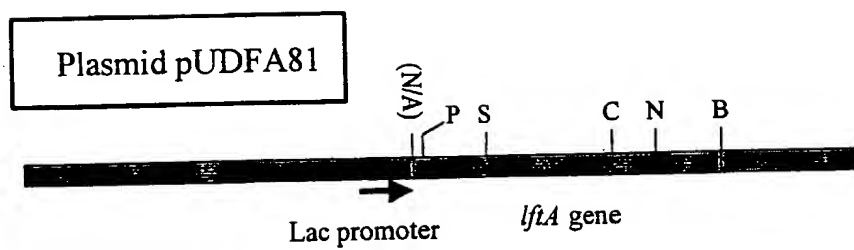
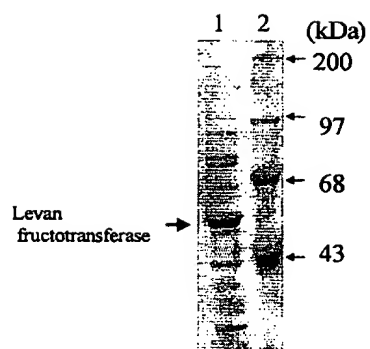


Fig. 8



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Fig. 9